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## New insights and changing paradigms in the regulation of vitamin A metabolism in development

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### Abstract

Vitamin A and its active metabolite retinoic acid are essential for embryonic development and adult homeostasis. Surprisingly, excess or deficiency of vitamin A and retinoic acid can cause similar developmental defects. Therefore, strict feedback and other mechanisms exist to regulate the levels of retinoic acid within a narrow physiological range. The oxidation of vitamin A to retinal has recently been established as a critical nodal point in the synthesis of retinoic acid, and over the past decade, RDH10 and DHRS3 have emerged as the predominant enzymes that regulate this reversible reaction. Together they form a codependent complex that facilitates negative feedback maintenance of retinoic acid levels and thus guard against the effects of dysregulated vitamin A metabolism and retinoic acid synthesis. This review focuses on advances in our understanding of the roles of *Rdh10* and *Dhrs3* and their impact on development and disease.

### INTRODUCTION

Vitamin A (retinol) is an organic nutrient that is essential for human embryonic development and adult homeostasis. More specifically, during embryogenesis, sufficient levels of vitamin A are critical for brain, craniofacial, limb, and organ patterning and morphogenesis.<sup>1–3</sup> Later, in adulthood, vitamin A plays important roles in fertility, immune function, maintenance of vision, and skeletal homeostasis.<sup>4–6</sup> Vitamin A is available to the human diet in two distinct forms: preformed vitamin A (retinol and its esterified form, retinyl ester) and provitamin A carotenoids. Provitamin A carotenoids serve as the principle source of vitamin A within the food chain and, include  $\alpha$ -carotene,  $\beta$ -carotene, and  $\beta$ -cryptoxanthin which are derived from plants. Preformed vitamin A is typically found in foods of animal origin, including dairy products, fish, and meat (especially liver) and most of the body's vitamin A is stored in the liver in the form of retinyl esters.

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Both provitamin A and preformed vitamin A are metabolized intracellularly via two sequential oxidation reactions to all-*trans*-retinoic acid (ATRA), the active form of vitamin A, which elicits essential biological functions. In the first reaction which is reversible, retinol is oxidized to form all-*trans*-retinal. In the second reaction, all-*trans*-retinal is irreversibly oxidized to ATRA. ATRA is a ligand for each of the three mammalian isotypes of the retinoic acid receptors (RARs), namely RAR $\alpha$ ,  $\beta$ , or  $\gamma$ ,<sup>7–10</sup> which form heterodimers with one of three different isotypes of the retinoid X receptor (RXR): RXR $\alpha$ ,  $\beta$ , or  $\gamma$ .<sup>11</sup> The RAR–RXR heterodimers associate with genomic elements consisting of direct repeats (DRs) termed retinoic acid response elements (RAREs) found in the promoter or vicinity of target genes (Figure 1).

In the classical model of retinoid signaling, RAR–RXR heterodimers associate with RAREs in the presence of corepressors, histone deacetylases, and methyltransferases, which maintain target genes in a transcriptionally silent state. Upon ATRA ligand binding, RAR–RXRs dissociate from corepressors and recruit coactivators resulting in transcriptional activation.<sup>12</sup> However, deviations from this classical model have been identified in which binding of ATRA to RAR–RXRs has been shown to recruit corepressors to silence gene activation. Repression of gene expression by retinoic acid was first identified in the restriction of the homeobox B1 (*Hoxb1*) gene.<sup>13</sup> Other deviations to the classical model have been found as in the case of *fibroblast growth factor 8* (*Fgf8*),<sup>14,15</sup> as well as, in the differential regulation of the paired homeobox proteins, by upregulation of PHOX2A and downregulation of PHOX2B.<sup>16</sup> Many of the genes controlled by RAR–RXRs play significant roles in cellular differentiation and developmental programming; thus, gene regulation by RAR–RXRs play critical roles in developmental patterning, differentiation of tissues, and organogenesis during embryonic development.<sup>1,3,17,18</sup> Furthermore, following birth, RAR–RXR-based signaling continues to play important roles in postnatal life in a wide array of processes such as the immune response, germ cell function, metabolic regulation, neuronal plasticity and tissue differentiation, regeneration, and repair.<sup>19–23</sup>

The mechanisms that regulate the formation of ATRA in embryos and the plethora of developmental processes in which ATRA plays a critical role have been the subject of several excellent and recent reviews.<sup>3,24–27</sup> However, the long established dogma that (1) oxidation of all-*trans*-retinol to all-*trans*-retinal was carried out solely by medium-chain alcohol dehydrogenase (ADH) enzymes, and did not serve an important role in the regulation of ATRA synthesis; and (2) that primary control of ATRA synthesis occurred solely at the second oxidative step has been overturned.<sup>28–30</sup> In the current review, we therefore first describe new developments and changing paradigms in our understanding of the feedback regulation of ATRA levels in embryonic tissues. Second, we focus on the new directions and ideas regarding the role of ATRA during developmental programming.

Morphogenesis and pattern formation during embryogenesis are driven by a multitude of signaling molecule gradients that orchestrate specific programs of gene expression and ultimately determine cell fate. ATRA signaling via RAR–RXR contributes to establishing cell fate by acting as a positional morphogen.<sup>31,32</sup> Information derived by an ATRA-responsive cell from the cellular levels of ATRA is integrated with positional and temporal

information derived from other morphogen gradients allowing the cell to make complex fate decisions.<sup>33</sup>

The actions of ATRA come from its function as an autocoid, acting near its site of synthesis due to its short half-life and relatively lipophilic composition. In many developmental processes, ATRA acts in a noncell autonomous fashion whereby the developmental fate of an ATRA-responsive cell population, which cannot synthesize ATRA, is regulated by a neighboring ATRA-synthetic cell population. Noncell autonomous modes of signaling for ATRA have been proposed in the case of the developing kidney<sup>34</sup> and brain vascular development.<sup>35</sup> In other processes, however, ATRA also acts in a cell-autonomous, autocrine fashion, that is, by directing the expression of genes within the same cell that synthesizes ATRA from a retinaldehyde precursor. For example, such autocrine effects of ATRA have been proposed in Sertoli cells.<sup>36</sup> This type of autocrine signaling by ATRA is particularly important in situations where ATRA regulates its own levels and metabolism within the ATRA-synthetic cell.

Regulating the levels of a diffusible molecule like ATRA requires the cooperation of binding proteins and transporters, and of synthesis and catabolism enzymes. The levels of ATRA within tissues need to be very tightly regulated to ensure proper signaling. The factors involved in ATRA metabolism and transport are modulated by both ATRA-dependent and by ATRA-independent pathways<sup>37</sup> and are responsive to the dietary or maternal levels of retinoid precursors.

It is important to consider that in nonlaboratory settings, the levels and/or composition of ATRA precursors such as preformed vitamin A and provitamin A carotenoids, vary considerably in the diet of different populations and even among different individuals within the same population. For example, Western diets are more reliant on animal products, which provide more preformed vitamin A and less provitamin A carotenoids than the diets available to the rest of the world's populations.<sup>38,39</sup>

Vitamin A deficiency can have serious deleterious effects ranging from loss of vision, to impaired growth and risk of infection.<sup>40</sup> Thus optimal levels of ATRA need to be maintained despite large individual differences in the absorption and conversion of provitamin A carotenoids.<sup>41</sup> Ensuring that embryonic tissues receive appropriate levels of ATRA at each particular stage and time of development depends on cooperation between both maternal and fetal retinoid metabolic pathways and their ability to adapt to the availability of vitamin A, as well as the need for ATRA.

## TERATOGENICITY OF EXCESS OR DEFICIENCY OF ATRA

A sufficient intake of vitamin A is critical for normal embryonic development;<sup>42–45</sup> however, administration of excess vitamin A or ATRA during early pregnancy can lead to congenital malformations of the head, heart, and limbs.<sup>46–50</sup> Hypervitaminosis A has also been associated with abnormal conditions in adults such as an increased risk of bone fractures in the elderly,<sup>51–53</sup> and in addition to teratogenic effects, pharmacological doses of ATRA can also cause toxicity associated with symptoms of retinoic acid syndrome.<sup>54</sup>

Paradoxically, similar congenital malformations have been observed in mothers that have ingested excess amounts of vitamin A during pregnancy or patients exposed to drugs based on ATRA or RAR-agonists, as in vitamin A-deficient patients and animal models.<sup>55,56</sup> The overlapping spectrum of congenital malformations and conditions irrespective of excess or deficiency in vitamin A or ATRA is due to the fact that changes in the levels of embryonic ATRA alters basic developmental and homeostasis pathways.<sup>57</sup>

The spatiotemporal levels of ATRA in an embryo following adult exposure to a pharmacological dose of ATRA, is subject to the time of exposure, the efficiency of transport of ATRA across the placenta, and the pharmacokinetics and distribution of ATRA in each tissue of the fetus. While in the past, exogenous ATRA administration to pregnant female mice was used to examine the effects of excess levels of embryonic ATRA, a recent study of the immediate and late effects of ATRA administration on mouse fetal development has painted a more complex picture. Even though a pharmacological dose of ATRA can initially result in an excess of ATRA in the embryo, this excess is followed by induction of ATRA-deficiency in the recovery period.<sup>58</sup> More specifically, within 6 h postexogenous administration of ATRA to the mother, the levels of ATRA in mouse embryos can be found in 1800-fold excess compared to untreated embryos. However, after an additional 6 h, the levels of embryonic ATRA diminish to the extent they become equal in treated versus untreated embryos. The embryonic levels of ATRA then continue to fall in treated embryos, such that by 24 h they are up to 33% lower than normally seen in untreated embryos.

The progression from short-term excess to long-term deficiency following administration of exogenous ATRA is most likely a result of the increased clearance of ATRA and compensatory enzymatic activities by maternal and embryonic tissues trying to cope with excess levels of ATRA. Similar, compensatory activity is also observed following genetic deletion of the different RARs which leads to the generation of teratogenic levels of ATRA.<sup>59</sup> Therefore, the pharmacokinetic profile of an acute dose of ATRA consists of a short period of ATRA excess in the embryo immediately after administration, followed by a long state of ATRA deficiency.

The surprising finding that many of the deleterious effects of exogenous ATRA administration could be corrected by a subsequent dose of ATRA<sup>58</sup> led to the conclusion that prolonged ATRA deficiency which follows an acute excess of ATRA, is responsible, at least in part, for many of the deleterious effects caused by exposure to pharmacological doses of ATRA. In fact, it is evident that ATRA deficiency induced by ATRA administration is more prolonged and has more toxic effects, than an acute change in the hours immediately following administration. Even modest changes in the embryonic levels of ATRA can result in developmental defects if these perturbations persist for a sufficiently long period of time. For example an increase in the steady state level of ATRA of only 40% in early mouse embryos results in teratogenic effects through E14.5. Similarly, decreases in the steady state levels of endogenous ATRA of comparable magnitude also elicit developmental defects.<sup>25,60,61</sup>

## MOLECULAR DETERMINANTS OF RETINOID METABOLISM IN EMBRYONIC TISSUES

### Uptake and Transport of Retinoids and Carotenoids

Provitamin A carotenoids are a significant source of dietary vitamin A in humans.<sup>39</sup> Carotenoids are absorbed in the intestine and are transported in the circulation by association with lipoproteins, mostly chylomicrons.<sup>62,63</sup> Carotenoids are taken up by several organs and tissues such as the liver, eye, ovaries, and fat. Although embryonic tissues primarily receive preformed vitamin A, there is evidence that provitamin A carotenoids can also be converted to ATRA within the fetus.<sup>60</sup> Provitamin A carotenoids, which represent carotenoids with one unsubstituted  $\beta$ -ionone ring, are oxidatively cleaved to produce retinaldehyde by the enzyme  $\beta$ -carotene-15,15-dioxygenase 1 (BCDO1; Figure 1).<sup>64–66</sup> BCDO1 is expressed at high levels in the enterocytes of the intestine and also in other tissues such as hepatic stellate and parenchymal cells in the liver, in mammary tissue, keratinocytes, and the kidney, as well as in embryonic tissues.<sup>67–69</sup> The expression of BCDO1 is negatively regulated by the intestine-specific transcription factor ISX which itself is induced by ATRA via RAR.<sup>70–73</sup>

Preformed vitamin A (retinol and retinyl esters) is hydrolyzed in the intestinal lumen by several lipases with broad specificity and then is taken up by enterocytes through facilitated transport or passive diffusion (reviewed in Refs 74 and 75). Retinol is re-esterified within the enterocytes primarily through the actions of lecithin retinol acyltransferase (LRAT).<sup>76</sup> Within the enterocyte, esterification of retinol is also enhanced in the presence of cellular retinol-binding protein II (CRBP2), which binds retinol with high affinity at low nanomolar concentrations.<sup>76–79</sup> Postprandial retinyl esters and carotenoids are secreted from the intestine as part of chylomicrons, the bulk of which are taken up by liver as chylomicron remnants. However, chylomicrons can also deliver retinoids to some target tissues including the embryo via the placenta.<sup>80–82</sup> Circulating carotenoids and retinyl esters are also found associated with lipoproteins including very low density lipoproteins.<sup>83,84</sup> Retinyl ester-containing chylomicron remnants are endocytosed by hepatocytes and hydrolyzed through the activity of several carboxylesterases and lipases.<sup>84,85</sup> Other ester hydrolases found in target tissues, such as lipoprotein lipase, hydrolyze retinyl esters from chylomicrons and allow cells to take up the free retinol.<sup>82,86</sup> It is important to note that a significant amount of unesterified retinol is secreted in the portal circulation independent of apolipoproteins and that this pathway may be important in certain pathological conditions.<sup>74</sup>

The main mode of transport for retinoids is via blood circulation and the most efficient delivery system for retinoids to embryonic tissues is in the form of retinol associated with serum retinol binding protein (RBP), or more specifically RBP4 (Figure 1). During postnatal life, RBP4 is secreted by hepatocytes which transport retinol to most target tissues including the placenta. Within the fetus, RBP4 is secreted by both fetal hepatocytes and the visceral yolk sac which facilitates the transport of retinol from the placenta to the embryonic sites of ATRA synthesis.<sup>87,88</sup> The importance of RBP4 was demonstrated in adult mice carrying *RBP4* null mutations as they displayed decreased fertility as well as severe structural changes to the retina with accompanying decreases in vision.<sup>89,90</sup> The role of RBP4 in delivering retinol to the placenta and from the placenta to embryonic tissues is evident in the

developmental delay and abnormalities associated with impaired synthesis of either maternal and/or the fetal-derived RBP.<sup>81</sup>

RBP interacts with several surface receptors on target tissues. The best known RBP receptor is stimulated by retinoic acid 6 (STRA6) which is found in many organ–blood barriers, in the retinal pigment epithelium (RPE), choroid plexus, and Sertoli cells, as well as epidermal keratinocytes, dermal fibroblasts, and the placenta.<sup>91–94</sup> STRA6 plays a critical role in allowing tissues such as the RPE and the choroid plexus to import retinol from the retinol–RBP complex referred to as holo-RBP.<sup>93,95–97</sup> Although it was previously suggested that STRA6 couples with CRBP1 to internalize retinol,<sup>98,99</sup> a recently determined cryo-EM structure of STRA6 suggests that STRA6 mediates internalization of retinol by diffusion through the cellular membrane.<sup>100</sup> In cases where there is an excess of serum RBP unbound to retinol, or apo-RBP, and unesterified retinol within the cytoplasm, STRA6 can mediate efflux of cellular retinol to apo-RBP (Figure 1).<sup>99,101,102</sup> A second RBP4 receptor (RBPR2) is found in the small intestine and hepatocytes, and is thought to potentially play a role in the reverse transport of retinol to the liver.<sup>103</sup>

The exact mechanism for maternal-fetal transfer of retinol from holo-RBP4 across the placenta to the developing fetus remains to be determined. Importantly, however, it has been shown that neither maternal, nor fetal RBP4 can cross the placenta.<sup>104</sup> There is clear evidence that the delivery of retinol via holo-RBP4 to the placenta is receptor-mediated<sup>105–107</sup> and that STRA6 is one possible placental RBP receptor.<sup>108</sup> But what mediates the transfer of retinol from the maternal placenta to the embryonic circulation is currently unknown. Similarly, it is also not known whether this transfer involves a transcellular or a paracellular pathway, or both.

Among the many issues and unanswered questions regarding the targeting and transport of RBP4 across the placenta, one of the most important is the relevance of extrapolating findings derived from mouse models to human biology. For example, there are important differences between rodents and humans in terms of placental development and even in terms of the role of the visceral yolk sac which functions throughout murine pregnancy but is mostly a vestigial structure in humans after the first trimester.<sup>109,110</sup> There are also species-specific differences in the extent of retinoid delivery to the fetus mediated by lipoprotein-based transport of retinyl esters. Therefore beyond initial reports,<sup>111,112</sup> there has been a paucity of studies investigating the mechanisms of retinol delivery across the human placenta.

What we do know is that LRAT is critically required for the uptake of retinol from plasma by target tissues such as RPE cells through binding to Stra6.<sup>113</sup> LRAT also plays an important role in the formation of retinyl esters in quiescent hepatic stellate cells, the body's principal vitamin A storage site under normal physiological conditions (Figure 1). Although stellate cells represent approximately 8% of the total cells in a healthy liver, they store 80–90% of the liver's retinoid content which is equivalent to 50–80% of the total retinoid stores of the body.<sup>114</sup> As in the intestine, esterification of retinol in the liver and in the RPE is enhanced in the presence of cellular RBPs, in this case, CRBP1.<sup>115–117</sup> Hydrolysis of retinyl esters stored in the liver or in target tissues can be carried out by several potential enzymes.



Of note, hormone-sensitive lipase and patatin-like phospholipase domain-containing 3 - (PNPLA3) have been implicated in the hydrolysis of retinyl esters in adipose tissue and hepatic stellate cells, respectively (Figure 1).<sup>118–120</sup> However, it is likely that there are several other enzymes capable of such activity in adipose tissue, hepatic stellate cells, and RPE.

### Regulation of Vitamin A Uptake, Transport, and Storage

In the case of circulating forms of retinoids, ATRA controls the expression levels of the receptors of serum RBP, namely, STRA6 and RBPR2. While the expression of STRA6 is induced by ATRA via RAR $\gamma$ ,<sup>121–123</sup> the expression of the liver RBP4 receptor, RBPR2, is repressed by ATRA and is negatively correlated with liver retinoid status.<sup>103</sup> ATRA also controls the expression levels of CRBP1 and CRBP2 which play important roles in channeling ligands to and from cellular receptors and/or enzymes.<sup>124,125</sup> In general, the expression of both CRBP1 and CRBP2 is increased in response to ATRA acting via RAR/RXR on several well-characterized RARE sequences found in their promoters. However, the levels of expression of CRBP2 in the small intestine are increased in the case of vitamin A deficiency, suggesting additional mechanisms may result in differential expression of these accessory proteins in specific tissues.<sup>126</sup>

Several mechanisms allow for cells to regulate the levels of retinyl esters to respond to vitamin A deficiency or to accommodate an intake of dietary vitamin A. Feedback regulation by ATRA signals vitamin A status and affects the uptake, delivery, and storage of retinol as retinyl esters.<sup>74</sup> ATRA augments the formation of retinyl esters through upregulation of LRAT.<sup>127,128</sup> These changes allow tissues to increase their storage of vitamin A in times of supply and also help tissues avoid forming excess ATRA from free retinol. The activity of BCDO1 also appears to regulate the activity and expression of LRAT and other acyltransferases and thereby influence the formation of retinyl esters in the fetus. Interestingly, the mechanism by which this occurs does not involve cleavage of its known substrate  $\beta$ -carotene.<sup>60,129</sup> Our knowledge of the intracellular retinyl ester hydrolases operating within the liver, RPE, adipose tissue, and other tissues to store retinoids remains rudimentary. However, retinyl ester hydrolases play a critical role in the uptake of retinol from retinyl esters as well as in determining the availability of retinol for ATRA. Thus, this process is likely to be tightly regulated. In support of this idea, the expression of the retinyl ester hydrolase, PNPLA3 in hepatic stellate cells, is inversely correlated with unesterified retinol availability.<sup>120</sup>

### Interconversion of Retinol and Retinaldehyde

The first step in the metabolism of vitamin A to ATRA is the oxidation of all-*trans*-retinol to all-*trans*-retinal (Figure 1). For many years, it was commonly thought that cytosolic ADHs were primarily responsible for this reaction during embryogenesis and that they carried out the reaction ubiquitously and redundantly. The primary reasons for this idea lay firstly in the observation that *Adh7* was ubiquitously expressed during embryogenesis. Secondly, although *Adh1* and *Adh4* exhibit tissue specific domains of activity,<sup>130</sup> *Adh1*<sup>−/−</sup>, *Adh4*<sup>−/−</sup>, and *Adh7*<sup>−/−</sup> loss-of-function mouse models survive into adulthood without evidence of developmental defects.<sup>131–133</sup> The lack of a discernible developmental phenotype in *Adh*

loss-of-function models therefore led to the idea that primary control for the synthesis of ATRA occurred at the second step through retinaldehyde dehydrogenase (RALDH) oxidation of retinal to ATRA. In support of this idea, *Raldh2* loss-of-function mouse mutants exhibited diminished ATRA synthesis in association with considerable developmental defects in heart, limb, and axial development.<sup>134</sup>

However, the long established dogma that (1) oxidation of all-*trans*-retinol to all-*trans*-retinal during embryogenesis was carried out solely by ADH enzymes and did not serve an important role in the regulation of ATRA synthesis; and (2) that primary control of ATRA synthesis occurred solely at the second oxidative step was overturned by the identification of the short-chain dehydrogenase, RDH10, and its role in the first oxidation step of vitamin A metabolism.<sup>28–30</sup> The importance of RDH10 in embryonic development and ATRA synthesis and signaling was clearly revealed in hypomorphic *Rdh10<sup>trax/trax</sup>* and null *Rdh10<sup>-/-</sup>* mouse models which are embryonic lethal between E10.5 and E14.5 in association with insufficient ATRA production.<sup>28–30,129</sup> Analysis of *in vivo* ATRA activity in null *Rdh10<sup>-/-</sup>* mutants carrying the *RARE-lacZ* transgene reporter, revealed a complete absence of RA production prior to E9.5.<sup>29</sup> Similarly, In *Rdh10<sup>trax/trax</sup>* embryos, ATRA activity was completely absent in the craniofacial, trunk, and limb regions. Only a considerably diminished domain of activity remained within the neural tube (Figure 2).<sup>28,136,137</sup> Furthermore, in enzymatic assays, the *Rdh10<sup>trax</sup>* mutant allele was shown to be incapable of oxidizing vitamin A (retinol) to retinal.<sup>28</sup> A further indication of RDH10's critical role in this process is the substantial reduction of NAD-dependent retinol oxidase activity in *Rdh10* loss-of-function embryos.<sup>30</sup> RDH10 is membrane bound in target cells and carries out the oxidation of all-*trans*-retinol within pools of the cellular membrane that are free of inhibitory RBP1.<sup>138</sup>

During embryogenesis, *Rdh10* is dynamically expressed both temporally and spatially commencing around E8.0 within the prospective hindbrain and lateral mesoderm tissues.<sup>28</sup> Between E8.25 to E10.5, *Rdh10* continues to be expressed in the lateral plate mesoderm, and dramatically expands to the somites, limb buds, head mesenchyme, primitive olfactory, optic, otic, lung, and foregut tissues.<sup>28</sup> The spatiotemporal pattern of *Rdh10* expression closely correlates with the spatiotemporal pattern of developmental defects observed in RDH10 loss-of-function mouse embryos. E9.5 *Rdh10<sup>trax/trax</sup>* mutants display duplicated or displaced otic vesicles and agenesis of the third to sixth caudal pharyngeal arches.<sup>28</sup> By E10.5, *Rdh10<sup>trax/trax</sup>* mutant embryos exhibit truncated forelimbs<sup>28,29</sup> with interdigital webbing that persists at E14.5<sup>136</sup> (Table 1). Somitogenesis, or the process of forming somites, which are precursors of metameric vertebrae and muscle, is also perturbed in *Rdh10<sup>trax/trax</sup>* mutants.<sup>140</sup> Furthermore, variable orofacial cleft phenotypes are evident in *Rdh10<sup>trax/trax</sup>* mutant embryos between E12.5 and E13.0 including a midline facial cleft of the frontonasal process in which the nasal septum and cavities fail to form.<sup>28</sup> In addition, these mutants exhibit disruptions in organogenesis with defective development of the heart, lungs, liver, stomach, pancreas, and gastrointestinal tract (Table 1).<sup>28</sup> *Rdh10<sup>-/-</sup>* null mutant embryos exhibit a more severe phenotype than *Rdh10<sup>trax/trax</sup>* embryos, which is consistent with a more complete loss of RA signaling.<sup>29</sup> By E9.5, null *Rdh10<sup>-/-</sup>* mutants exhibit defective turning, axial elongation, and overall growth. Furthermore, the first pharyngeal arch is reduced in size and arches 2–6 fail to form. *Rdh10<sup>-/-</sup>* null mutants exhibit embryonic



lethality by E10.5, likely as a consequence of severe cardiac defects including failure to undergo looping and failure to form the proper heart chambers.<sup>29</sup> Other more recently generated *Rdh10* loss-of-function mouse models have revealed critical roles for Rdh10 and RA signaling in salivary gland development, brain vascular formation, juvenile spermatogenesis, and dark vision adaptation<sup>35,145–147</sup> (Table 1).

Nonmammalian models have also contributed to our understanding of the role *Rdh10* plays during embryogenesis and in the production and regulation of ATRA. The characterization of these models has helped to demonstrate the conserved role of RDH10 within several vertebrate species during embryogenesis. In zebrafish, *rdh10a*, is expressed in the paraxial mesoderm in early somite stage embryos<sup>148</sup> and in the absence of *rdh10a*, zebrafish embryos exhibit a decrease in ATRA production and signaling. At 24 h postfertilization, this results in anteriorization of the nervous system, together with enlarged hearts and increased numbers of cardiomyocytes.<sup>144</sup> The phenotype is considerably worsened when *rdh10a* is depleted in combination with *aldh1a2* (*raldh2*), the primary enzyme responsible for the second oxidation step of vitamin A metabolism.<sup>144</sup>

Interestingly, depletion or inhibition of RA synthesis results in an increase in *Rdh10* expression.<sup>29,148,149</sup> Conversely, exposure to exogenous RA has the opposite effect of decreasing *Rdh10* expression. These findings are indicative of a negative feedback mechanism in which *Rdh10* is inactivated when sufficient levels of ATRA have been produced. In further support of this idea, *Rdh10* possesses a putative RARE in its 5' upstream enhancer control region suggesting this feedback may be direct.<sup>29</sup> Thus, loss-of-function studies in animal models support a role for RDH10 as the predominant and earliest acting enzyme in the oxidation of retinol to retinal during embryonic development. The interconversion of all-*trans*-retinol to all-*trans*-retinal by RDH10 is therefore now recognized as an important spatiotemporal control or nodal point in the synthesis of ATRA, which assists in regulating the levels of ATRA during embryogenesis to help guard against vitamin A toxicity.

Although RDH10 is currently considered the most important retinol dehydrogenase present in the early embryo, there are other enzymatic activities that contribute to retinaldehyde formation and retinoid function later in life. For example, two additional retinol dehydrogenases, RDH1 and DHRS9, play important roles in postnatal retinoid metabolism in adipose tissue and astrocytes, respectively.<sup>150,151</sup> Furthermore, several soluble enzymes belonging to classes I, III, and IV ADH enzymes have been shown to play a role in postnatal retinol metabolism by protecting against vitamin A toxicity.<sup>131,152–154</sup>

It is important to note that the oxidation of retinol to retinal is reversible and the reduction of all-*trans*-retinal to retinol is particularly important in the conversion of  $\beta$ -carotene to retinol for storage and transport to target tissues (Figure 1). Many retinaldehyde reductases have been identified in the eye (reviewed in Ref 155) and the reduction of all-*trans*-retinaldehyde is critical for vision.<sup>156</sup> However, despite the well-recognized roles for retinaldehyde reductases, the identity of individual reductases with physiological relevance in tissues, besides the eye, remain to be determined.

Recently, we and others described a role for the short-chain dehydrogenase/reductase (SDR) family, member 3 (DHRS3) in reducing retinaldehyde to retinol.<sup>135,139,141,157,158</sup> *Dhrs3* is expressed in a dynamic spatiotemporal pattern that overlaps with many tissues in which vitamin A metabolism and signaling are known to occur during mouse embryogenesis. The highest levels of DHRS3 protein in E14.5 embryos are observed in the liver, kidney, nasal epithelium, and interdigital zones of limbs (Figure 2). In the heart, high levels are found in the pericardial mesothelium, while in the neural tube, high levels of activity are found along the dorsal and ventral midline. In the brain, DHRS3 also localizes to the developing pituitary, cerebellum, and choroid plexus and has been observed in the retina, thyroid, lung, and intestine (Figure 2).<sup>135</sup> In addition to mouse embryos, DHRS3 is known to be active in human testes, liver, and small intestine tissues.<sup>157</sup>

Loss-of-function studies in mice have demonstrated that *Dhrs3*<sup>-/-</sup> is critical for normal embryogenesis as mutant embryos die during late gestation, around E17.5 to E18.5.<sup>135</sup> Similar to models of *Rdh10* loss-of-function, *Dhrs3*<sup>-/-</sup> mutant embryos display perturbed craniofacial, heart, and axial development (Figure 2 and Table 1).<sup>135</sup> In the head, the palatal shelves fail to elevate and then also fail to fuse, resulting in cleft palate. Cardiac anomalies present in the form of ventricular septal defects, atrial septal defects, and double-outlet right ventricle. In the axial skeleton, vertebral fusions, specifically at C1 and C2 occur, as well as delayed ossification within the jaw, and skull.<sup>135</sup> In *Dhrs3*<sup>-/-</sup> mice containing the *RARE-lacZ* transgenic reporter, ATRA activity was shown to be increased in the majority of tissues compared to wild-type littermates with expanded domains particularly in the frontonasal region and tail<sup>145,146</sup> (Figure 2). Notably, the *Dhrs3*<sup>-/-</sup> mutants which displayed the largest increase in ATRA activity tended to exhibit the most severe developmental defects.

In addition to mice, other species-specific models have validated the role of DHRS3 in vitamin A metabolism and embryogenesis providing evidence for its conserved role between vertebrate species. For example, a reduction of *dhrs3a* in zebrafish leads to increased ATRA-reporter activity and activation of ATRA-target genes.<sup>148</sup> More specifically, the knockdown of *dhrs3a* results in an expanded domain of ATRA signaling which encompasses the anterior portion of the spinal cord and part of the hindbrain including rhombomeres 7/8. This impacts nervous system patterning and results in a reduced number of neurons known as T-interneurons in the hindbrain.<sup>148</sup> Similarly, morpholino knockdown of *dhrs3* in *Xenopus* embryos also produced a phenotype characteristic of ATRA excess. *Xenopus dhrs3* morphants presented with smaller heads that were reduced in diameter. In addition, the mutants also exhibit altered neuroectoderm patterning as well as defects in somitogenesis, including diminished gene expression and perturbed segmentation.<sup>141</sup>

Although DHRS3 is the primary retinaldehyde reductase known to be present in embryonic tissues, it may not be the only retinaldehyde reductase to play a role in embryogenesis. Interestingly, residual retinaldehyde reductase activity can be detected in microsomes and mouse embryonic fibroblasts (MEF) derived from *Dhrs3*<sup>-/-</sup> mice.<sup>139</sup> Consistent with this observation, it is known that retinaldehyde reduction can also be carried out *in vitro* by several members of the aldo-keto-reductase (AKR) family. However, it remains to be determined whether AKR enzymes play such an *in vivo* role during embryogenesis.<sup>24,159–162</sup> Despite this uncertainty, it is clear DHRS3 has well-defined physiological

relevance based on the alterations in systemic ATRA metabolism, RAR-signaling, and developmental defects observed in *Dhrs3* mutant fish, amphibian, and mouse embryos.<sup>135,139,141,148</sup>

Although members of three distinct enzyme families have been implicated in the interconversion of retinol and retinaldehyde based on enzymatic activities demonstrated *in vitro*, only in limited instances has a definitive *in vivo* role in ATRA metabolism been corroborated by loss-of-function approaches. To date, RDH10 and DHRS3 are the only retinol/retinaldehyde oxidoreductases whose ablation has been shown to result in developmental defects. The lack of an apparent developmental phenotype (except in the eye) in animals deficient in other retinoid-specific SDR, ADH, or AKR enzymes does not rule out the possibility that additional enzymes play a role in retinol/retinaldehyde interconversion. Rather the indispensable role of RDH10 and DHRS3 during embryonic development could reflect the fact that they are expressed earlier than other potential retinol/retinaldehyde oxidoreductases, or that they are expressed in a tissue or tissues where no compensatory activities exist.

### Formation and Breakdown of ATRA

The second step in the metabolism of vitamin A to ATRA is the oxidation of retinal to ATRA. The conversion of retinaldehyde to ATRA is an irreversible reaction catalyzed by several retinaldehyde dehydrogenases ALDH1A1, ALDH1A2, and ALDH1A3 (formerly RALDH1, RALDH2, and RALDH3) which belong to the aldehyde dehydrogenase family.<sup>163–166</sup> Of these, ALDH1A2 appears to be the most broadly active during early embryogenesis.<sup>164,167</sup> ALDH1A2 plays a critical role in the conversion of retinaldehyde into ATRA in the spinal cord, developing heart, retina, lung, and inner ear olfactory epithelium.<sup>134,168–171</sup> *Aldh1a3* is expressed in sensory neuroepithelia and plays an important role in ATRA formation in the developing nasal structures and eye.<sup>172–175</sup> *Aldh1a1* is expressed in the developing neural retina, and many tissues after birth, however, a deficiency of *Aldh1a1* does not result in apparent developmental defects. Rather, *Aldh1a1* loss-of-function influences lipid metabolism in postnatal life in response to increased levels of retinaldehyde.<sup>167,176–181</sup> In addition to ALDH1A1–3, several cytochrome P450 enzymes, can oxidize retinaldehyde to ATRA, and CYP1B1 in particular has been shown to contribute to the formation of ATRA *in vivo*.<sup>182,183</sup>

### Regulation of ATRA Formation and Breakdown

The physiological levels of ATRA are tightly regulated to guard against the effects of either ATRA deficiency or excess. ATRA levels are modulated through a negative feedback mechanism, which serves to regulate ATRA-synthetic enzymes in the presence of elevated ATRA levels. Consistent with this mechanism *Aldh1a1*, *Aldh1a2*, and *Aldh1a3*<sup>184–186</sup> are downregulated in response to increasing concentrations of ATRA. Furthermore, RDH10, the principal enzyme responsible for oxidation of retinol to retinal during embryogenesis was also shown to be downregulated in response to exogenous ATRA or elevated endogenous levels of ATRA as seen in *Dhrs3*<sup>−/−</sup> mice.<sup>29,135,148,149</sup>

A critical regulatory control point in the metabolism of vitamin A and synthesis of ATRA occurs at the first step in the reversible interconversion of retinol and all-*trans*-retinal. The function of the oxido-reductase enzymes, RDH10 and DHRS3, is to help alleviate excess as well as deficient levels of ATRA. DHRS3, by reducing retinaldehyde, leads to a decrease in the ATRA precursor and, therefore, protects tissues from the formation of excess ATRA. The expression of DHRS3 is responsive to ATRA levels and creates a negative feedback loop to further restrict the formation of ATRA.<sup>141,148,187,188</sup> The oxidation of retinol to all-*trans*-retinal by RDH10 serves the reverse function of DHRS3 by protecting against a retinoid deficiency. This oxidative step can be modulated in the presence of excessive levels of ATRA by downregulating expression of RDH10,<sup>135</sup> which acts as part of the negative feedback mechanism to prevent further synthesis of ATRA. Conversely, in response to a deficiency in ATRA such as in the case of *Raldh2* loss-of-function, the activity of RDH10 can increase in an attempt to compensate.<sup>29</sup>

Another level of regulation in the interconversion of retinol to all-*trans*-retinal occurs through an interplay between RDH10 and DHRS3 which form a complex. RDH10 and DHRS3 are codependent as DHRS3 relies on RDH10 to maintain its full catalytic activity. Similarly, RDH10 requires interaction with DHRS3 for complete activation.<sup>139</sup> This interaction has been corroborated in cell culture studies by demonstrating colocalization of the two proteins. Additionally, *Rdh10* and *Dhrs3* exhibit overlapping regions of activity.<sup>28,135</sup> For example, *Rdh10* and *Dhrs3* have been shown to be expressed in the interdigital regions of the hindlimbs of E12.5–14.5 mouse embryos which supports the potential for their direct physical interaction.<sup>139</sup> However, future studies aimed at thoroughly characterizing the coexpression of these enzymes in tissues throughout embryogenesis are still critically needed.

The interaction and subsequent activation of DHRS3 by RDH10 acts to reduce the levels of excess ATRA. In MEF derived from *Dhrs3*<sup>-/-</sup> mutant embryos, the production of retinol from retinaldehyde is diminished, and furthermore, cells coexpressing both *Rdh10* and *Dhrs3* produce less retinaldehyde and ATRA.<sup>139</sup> Therefore, in the instance of excess ATRA, DHRS3, which is upregulated by ATRA, interacts with RDH10 enabling it to begin converting retinaldehyde back to retinol, thereby preventing its conversion by ALDH enzymes to ATRA. In the presence of RDH10, retinol may be converted back to retinaldehyde; however, the overall production of ATRA from retinaldehyde is greatly reduced in the presence of DHRS3, thereby protecting against ATRA excess. It is through this interplay between RDH10 and DHRS3, and the interconversion of retinol and retinaldehyde, that ATRA can be reduced in times of excess without stopping its production completely. This allows for the precise regulation of ATRA production, which helps guard against harmful teratogenic effects to the developing embryo.<sup>139</sup>

## CONCLUSIONS

Vitamin A (retinol) and its active metabolite ATRA are essential for normal embryonic development and adult homeostasis as defects can arise in these processes in association with either retinoid excess or deficiency. Therefore, in periods of both vitamin A and ATRA

excess or deficiency, compensatory mechanisms that regulate uptake, delivery and storage of retinol exist to maintain ATRA levels within a tight physiological range.

The oxidation of retinol to retinal and its reversible interconversion, which are mediated predominantly by RDH10 and DHRS3 respectively, have now been firmly established as critical control or nodal points in vitamin A metabolism and the synthesis of ATRA (Figure 1). Both RDH10 and DHRS3 are essential for embryonic development (Table 1) and together these enzymes form a codependent complex that helps to maintain appropriate levels of ATRA. It is through complex mechanisms including the precise regulation provided by RDH10 and DHRS3, that embryos and adults are protected against the potentially harmful effects of dysregulated levels of vitamin A and ATRA.

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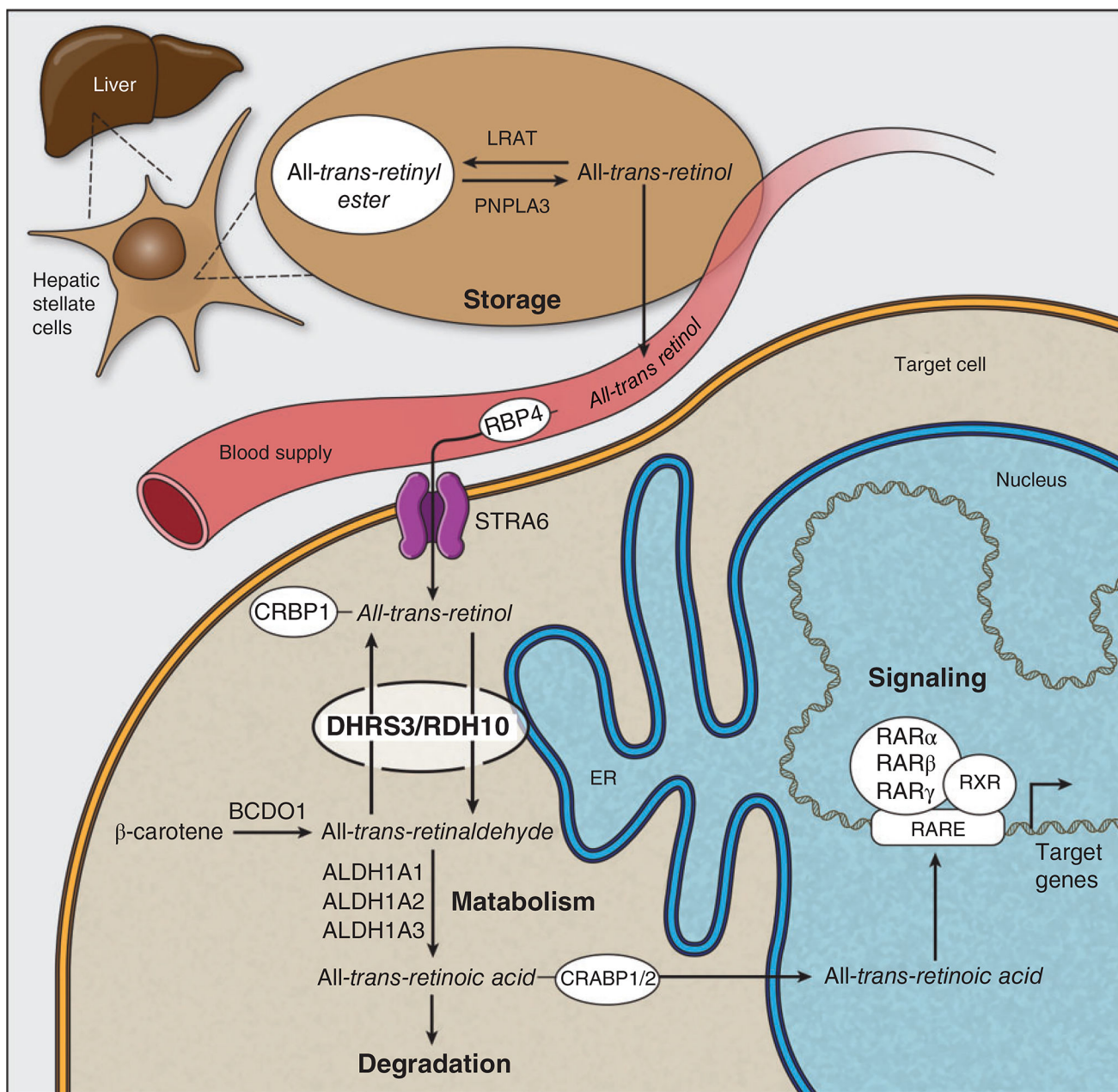
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**FIGURE 1.**

Preformed vitamin A is taken up from the intestinal lumen by enterocytes and transported via the blood supply as all-*trans*-retinol to its various target tissues. Large stores of vitamin A are kept in hepatic stellate cells of the liver by esterification of all-*trans*-retinol to retinyl esters. Mobilization of these stores is carried out by hydrolysis of retinyl esters back to all-*trans*-retinol where it is then transported by the blood supply through binding to RBP4. At target tissues, the RBP receptor, STRA6, allows for the import of retinol associated with RBP4. Once inside the cell, retinol associates with the RDH10/DHRS3 complex in the first reversible step of vitamin A metabolism to be oxidized to all-*trans*-retinaldehyde by RDH10. All-*trans*-retinaldehyde can then be reduced back to all-*trans*-retinol by DHRS3, or it may be

further oxidized by the ALDH genes (ALDH1A1–ALDH1A3) to form ATRA. ATRA then serves as a ligand for one of three isotypes of the RARs which form a heterodimer with RXR. The RAR–RXRs associate with retinoic acid response elements (RARE) within the promoters of target genes and can induce both transcriptional activation and silencing.

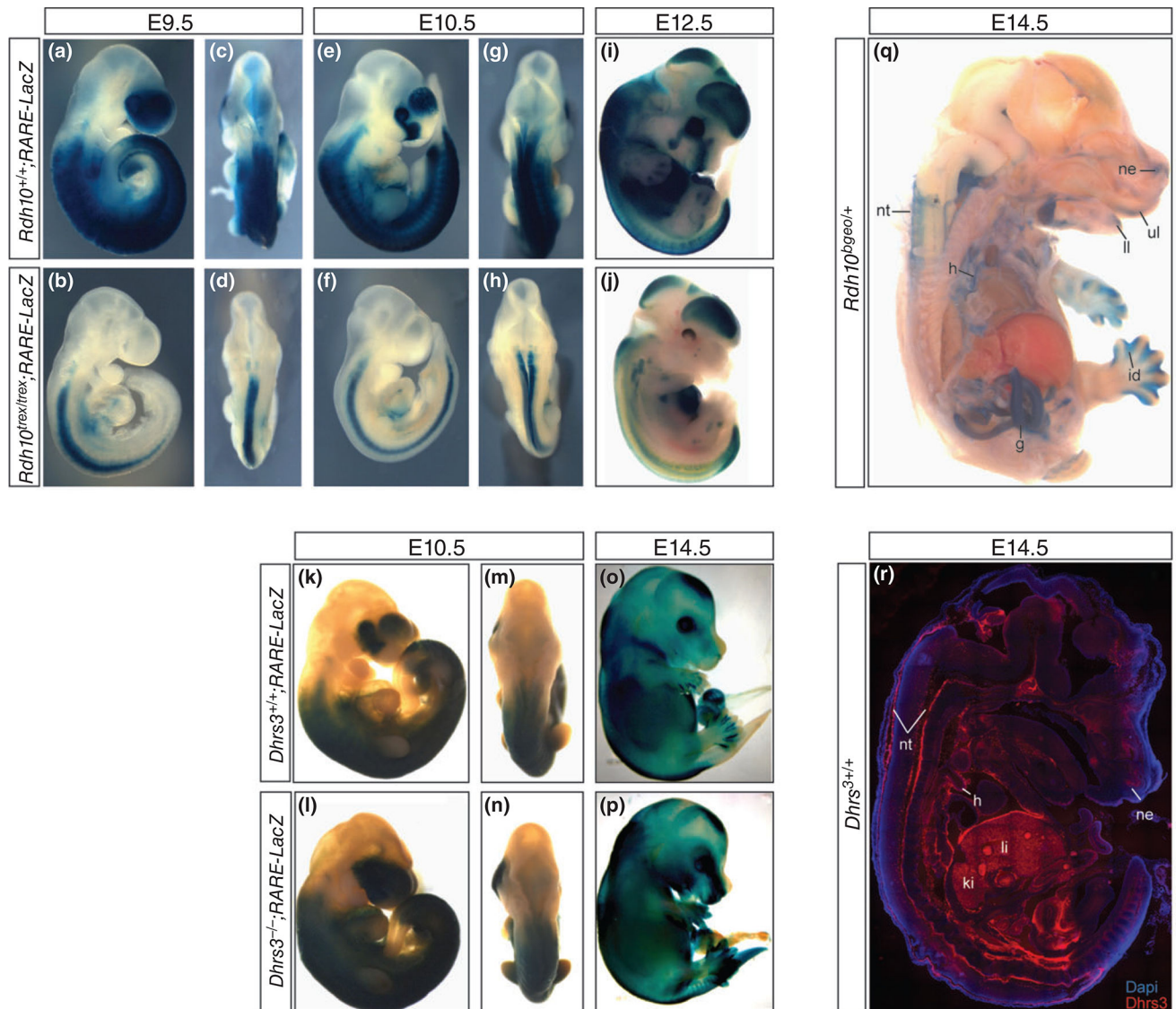
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**FIGURE 2.**

(a–p) Lateral and dorsal views of E9.5, E10.5, E12.5, and E14.5 control *RARE-LacZ*, *Rdh10*<sup>tex/trex</sup>; *RARE-LacZ*, and *Dhrs3*<sup>-/-</sup>; *RARE-LacZ* embryos illustrating diminished retinoic acid signaling in the craniofacial and trunk regions of *Rdh10*<sup>tex/trex</sup> embryos and expanded regions of signaling in the frontonasal region and tail of *Dhrs3*<sup>-/-</sup> embryos. (q) Lateral view of LacZ expression in a bisected E14.5 *Rdh10*<sup>bgeo/+</sup> embryo revealing RDH10 activity in the upper and lower lip, nasal epithelium, neural tube, heart, gut, and interdigital zone of the limbs. (r) Lateral view of an E14.5 embryo section immunostained with anti-DHRS3 (red) and DAPI (blue) illustrating DHRS3 activity in the neural tube, heart, liver, kidney, and nasal epithelium; g, gut; h, heart; id, interdigital zone of the limbs; ki, kidney; li, liver; ul, upper lip; ll, lower lip; nt, neural tube; ne, nasal epithelium. Panels (g)–(i) were adapted from Ref 135.



TABLE 1

Recorded Developmental Defects for Animal Models of *Rdh10* and *Dhrs3* loss-of-function

| Phenotype        | <i>Rdh10</i>  | <i>Dhrs3</i>   |
|------------------|---|--|
| Lethality        | <i>Rdh10<sup>flox/flox</sup></i> E10.5 to E14.5 <sup>28</sup><br><i>Rdh10<sup>-/-</sup></i> E10.5 <sup>28</sup>   | <i>Dhrs3<sup>-/-</sup></i> E17.5 <sup>135</sup>  |
| Craniofacial     | Clefting<br><i>Rdh10<sup>m366Asp</sup></i> E10.5 midline facial cleft <sup>61</sup><br><i>Rdh10<sup>flox/flox</sup></i><br>E12.5–E13.0 frontonasal process displays variable clefting <sup>28</sup><br>E12.5–E13.0 defective formation of the nasal septum and chambers <sup>28</sup>   | Palatogenesis<br><i>Dhrs3<sup>-/-</sup></i><br>E14.5 palatal shelves fail to elevate <sup>135</sup><br>E18.5 clefting of the secondary palate <sup>139</sup> |
| Somites          | Somitogenesis<br><i>Rdh10<sup>flox/flox</sup></i> 8–15 somite stage consistently smaller somites 1–6; somite size normalizes from somite 7 onward <sup>140</sup>  | Somitogenesis<br>Xenopus defective somitogenesis <sup>141</sup>  |
| Skeletal         | <i>Rdh10<sup>flox/flox</sup></i> E14.5 lack atlas and axis vertebrae <sup>140</sup>   | Axial skeleton<br><i>Dhrs3<sup>-/-</sup></i> E14.5/E17.5 abnormal axial skeleton development; vertebral fusions and delayed ossification <sup>135</sup>      |
| Limb development | Forelimb growth<br><i>Rdh10<sup>flox/flox</sup></i> and <i>Rdh10<sup>-/-</sup></i> E10.5–E13.0 stunted forelimb growth, however, normal hindlimb morphology <sup>3,28,29,61,142</sup><br>Interdigital webbing<br>E14.5 fail to lose interdigital mesenchyme <sup>61,136</sup>   | No recorded developmental defects  |
| Brain            | Hindbrain patterning <i>Rdh10<sup>-/-</sup></i> E7.75 defective hindbrain patterning <sup>142,143</sup><br>Brain Vasculature<br><i>Rdh10</i> ENU induced mutant E14.5 long, thin neocortex with reduced numbers of blood vessels. Large diameter blood vessels in PNVP vasculature <sup>35</sup>  | No recorded developmental defects  |
| Organogenesis    | Heart<br><i>Rdh10<sup>flox/flox</sup></i> E9.5 heart edema and looping effects <sup>28</sup><br><i>Rdh10<sup>-/-</sup></i> fail to undergo looping and chamber formation <sup>29</sup><br>Xenopus enlarged heart with increased cardiomyocyte number <sup>144</sup><br>Eyes<br>RPE-specific <i>Rdh10</i> knockout at 6wks impairs in 11- <i>cis</i> -retinal regeneration and effected dark adaptation after bright illumination <sup>145</sup><br>Lungs<br><i>Rdh10<sup>flox/flox</sup></i><br>E10.5 lung bud agenesis <sup>28</sup><br>E12.5 to E13.0 hypoplasia <sup>28,142</sup><br>Liver<br><i>Rdh10<sup>flox/flox</sup></i> E12.5 to E13.0 hypoplastic with fewer lobes <sup>28,142</sup><br>Pancreas<br><i>Rdh10<sup>flox/flox</sup></i> E12.5 to E13.0 structures not found <sup>28,142</sup> | Heart<br><i>Dhrs3<sup>-/-</sup></i> defective cardiac outflow tract formation; defects in atrial and ventricular separation <sup>135</sup>                   |

| Phenotype | <i>Rdh10</i>  | <i>Dhrs3</i> |
|-----------|---|--------------|
|           | Salivary gland  |              |
|           | <i>Rdh10<sup>prex/trex</sup></i> E14.5 size reduction in submandibular salivary glands; normal morphology <sup>146</sup>  |              |
|           | Stomach/Midgut  |              |
|           | <i>Rdh10<sup>prex/trex</sup></i> E12.5 to E13.0 hypoplastic <sup>28</sup>   |              |
|           | Testes  |              |
|           | <i>Rdh10<sup>fl/fl</sup></i> , Amh-Cre <sup>+</sup> juvenile male mice at 2–3 weeks defective sertoli cell differentiation of spermatogonial cells <sup>147</sup> |              |

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